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Research Article

Evaluating the potential of DNA metabarcoding for ecological status assessment under the Water Framework Directive: A case study on benthic invertebrates from Western Carpathian streams

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Abstract

Routine biomonitoring under the Water Framework Directive (WFD) follows a conventional methodology primarily based on the morphological identification of taxa within the five biological quality elements (phytoplankton, phytobenthos, macrozoobenthos, macrophytes and fish). This identification is particularly challenging for macrozoobenthos due to their high taxonomic diversity. Moreover, this approach is time-consuming and resource intensive. Our current study aims to: (i) evaluate the implementation of DNA metabarcoding in long-term monitored sites to assess the effectiveness of three sample types (bulk samples of macrozoobenthos, and eDNA from water and sediments), (ii) evaluate the ecological status of water bodies using metabarcoding data, and (iii) compare ecological metrics values using conventional and molecular approaches. Sampling was conducted at 17 localities in Slovakia, covering eight stream types. DNA metabarcoding detected 30% more species than conventional monitoring performed over 15 years without needing time-intensive sample processing and morphological identification. At the same time, the bulk samples captured more macrozoobenthos taxa than the other sample types. On average, the bulk samples detected 85% of the taxa captured by DNA metabarcoding. The differences in values of ecological metrics obtained within the conventional approach and between the conventional and metabarcoding approaches were relatively small, typically corresponding to differences within one ecological quality class. However, more pronounced differences were observed in non-abundance-based metrics, such as EPT and BMWP, indicating higher sensitivity of species detection with DNA metabarcoding. This study demonstrates the effectiveness of DNA metabarcoding across multiple metrics and highlights its potential to enhance routine biomonitoring.

Key words: Biomonitoring, bulk samples, eDNA, morphological approach, macrozoobenthos, Slovakia

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Introduction

The earliest protocols for assessing aquatic ecosystems were developed in the mid-19th century (Cohn 1853), but their practical application only began about a half-century later (Kolkwitz and Marsson 1902, 1908, 1909). In the second half of the 20th century, many indices using benthic macroinvertebrates were introduced to assess the structural and functional integrity of surface waters (Wiederholm 1980; Sládeček et al. 1981; Sládečková and Sládeček 1994; Rosenberg and Resh 1993; Hawkes 1998), many of which have become integral to national standards and legislation for assessing water quality across several countries (e.g., Barbour et al. 1992; Hering et al. 2004a, b; Ofenböck et al. 2004). Regarding water-quality assessment in Europe, it culminated with the adoption of the EU Water Framework Directive (WFD) in 2000, which established a harmonised framework to assess and prevent the deterioration of Europe's rivers, lakes and groundwater (European Commission 2000). This significantly changed the approach of Member States and some non-EU countries to water resources policy by prioritising ecosystem integrity in decision-making.

Today, more than 110,000 surface water bodies are assessed at regular six-year intervals in Europe, with their quality expressed by ecological status or potential, using physico-chemical and hydro-morphological parameters and biological indicators (Fueyo et al. 2024a; Kaavi and Paloniitty 2024). Within these, the WFD requires the establishment of reference conditions for each type that represent a state with minimal or no anthropogenic disturbance, from which the assessment of other monitored water bodies is then derived (Jupke et al. 2022). Among biological indicators, or biological quality elements (BQEs), macrozoobenthos is the most used group (Birk et al. 2012). This is also supported by recent data from the European Environment Agency's Waterbase dashboard, which shows that macrozoobenthos is consistently monitored across European surface water bodies (European Environment Agency 2024; for more details, see: https://water.europa.eu/freshwater/resources/metadata/wfd-dashboards/surface-waters-ecological-status). Following morphological identification, the species composition is used to calculate indices that are subsequently compared to reference values defined for each water body type. The ecological quality ratio (EQR), derived from this process, is reflected in the ecological status class (EQC) 1–5 (Fueyo et al. 2024a). However, sampling methods and the set of metrics used for ecological assessment vary considerably across countries, and the choice of metrics can also differ between regions and stream types within the same country (Birk et al. 2012; Buss et al. 2015; Makovinská et al. 2015, 2021). For example, in Slovakia, the ecological status of water bodies based on macrozoobenthos is assessed using a multimetric index (MMI) composed of a set of metrics, whose number and selection depend on the stream type and categories divided according to ecoregion (Carpathian, Pannonian), elevation (up to 200 m, 200–500 m, 500–800 m, above 800 m a.s.l.), and catchment area size (small, medium, large). In this context, Slovakia defines 24 stream types, with the set of selected metrics included in the MMI ranging from 6 to 11 metrics (Makovinská et al. 2015). Despite its use, the current methodology is both time- and cost-intensive, with several potential shortcomings, often related to the precise morphological identification of organisms, which can be affected by phenotypic

plasticity at different developmental stages or the loss of key morphological features in damaged specimens, as well as the increasingly limited availability of taxonomic experts (Bush et al. 2019; Jerde, 2021; Van den Bulcke et al. 2024; Páll-Gergely et al. 2024; Elbrecht and Leese 2015; Pinna et al. 2024; Zhang et al. 2023). Moreover, in Slovakia, another key issue is sub-sampling, in which only a fraction of the collected sample, typically around 20% (depending on organism density) is analysed and subject to morphological identification (Makovinská et al. 2015), which can lead to the omission of rare, low-abundance species and underestimate the present biodiversity.

Recent advancements in macrozoobenthos analysis have been driven by the adoption of molecular techniques, particularly bulk sample DNA and environmental DNA (eDNA) metabarcoding, which have helped to overcome the limitations of conventional methods (Tzafesta et al. 2021; Pinna et al. 2024). These techniques have the potential to reduce costs and processing time (Sepulveda et al. 2020; Zhang et al. 2023), while also facilitating the identification of juveniles, early instars, mechanically damaged individuals, or cryptic species (Thomsen et al. 2012; Bohmann et al. 2014; Serrana et al. 2019). Some studies have also highlighted the potential of sample fixative from preserved individuals for subsequent identification (Zizka et al. 2019; Vargovčík et al. 2024). Metabarcoding techniques can contribute to the discovery of new species (Nardi et al. 2020) and may assist in the detection of non-native, invasive (Nester et al. 2020; Sepulveda et al. 2020) and protected species (Fueyo et al. 2024b). In particular, eDNA metabarcoding can enable broader ecological monitoring across larger geographic areas, which enhances the effectiveness of national monitoring programs (Poyntz-Wright et al. 2024). Although such monitoring offers a more comprehensive perspective on the ecological status of water bodies, it also is not without limitations. The persistence of DNA released by organisms plays a crucial role, while it varies depending on the taxa and environmental conditions (Tsuri et al. 2021; Mauvisseau et al. 2022). Moreover, the temporal and spatial scale of the eDNA signal remains difficult to clearly define due to the complex ecology of eDNA, which is influenced by numerous biological and environmental factors (Barnes and Turner 2016; Mauvisseau et al. 2022). eDNA analysis can sometimes reveal taxa that are no longer present at the sampling site or those originating from upstream locations (Fonseca et al. 2023). One of the most significant and recurring limitations of using metabarcoding approaches in the monitoring of waters is the determination of the species abundance, which is used for calculating abundance-dependent metrics (e.g., saprobic index, bioecological area index). Some studies have addressed this challenge by transforming abundance data into presence/absence data (Buchner et al. 2019) or using the number of sequences reads as a proxy for abundance, though this approach remains quite disputable (Elbrecht et al. 2021; Macher et al. 2021a).

The potential of using DNA-based methods in the assessment of biological quality elements of water is considerable. We aimed to evaluate the potential of DNA metabarcoding under the real conditions of long-term monitored sites in Slovakia. Specifically, we set out to (i) assess the effectiveness of DNA metabarcoding in detecting taxonomic diversity across three sample types (bulk samples of macrozoobenthos, and eDNA from water and sediments), (ii) compare the taxonomic composition obtained through

conventional and molecular methods, and (iii) evaluate selected ecological metrics and the overall ecological status derived from metabarcoding data, using read counts as a proxy for abundance. We hypothesized that DNA metabarcoding would reveal higher species diversity than traditional methods, affecting some, but especially abundance-dependent metrics.

Material and methods

Data collection

The material was collected at 17 WFD sampling sites in Slovakia in 2022 and 2023 at elevations ranging from 147 to 905 m a.s.l. (Fig. 1). These localities represent 8 different stream types defined in Šporka et al. (2009). Metabarcoding analyses included samples from water, sediments, and bulk samples. Water samples were collected along a transverse transect covering the entire stream width using a sterile one-liter container until a total volume of 10 L was obtained in a sterile vessel. Water was usually taken from the water column (in smaller streams only from the surface layer), while avoiding disturbance of bottom sediments. From the collected volume, one-liter of water was filtered using disposable sterile 50 mL syringes through a filter holder (Swinnex Filter Holder, 47 mm) containing a cellulose filter with a pore size of 0.45 µm. This procedure was repeated twice at each site (two replicates of eDNA water samples). Filters were immediately placed in 96% ethanol in the field and stored in a cooling box until transport to the laboratory, where they were kept at −25 °C until further processing. Sediments were also collected in two replicates using sterile 50 ml Falcon tubes. Each replicate was taken from a different microhabitat type on the riverbed: one from a riffle (shallow, fast-flowing section) and the other from a pool (deeper, slower-flowing section) of the stream. Approximately 25 ml of sediment was collected per replicate, and after sediment settling, excess water was removed. The sample was then supplemented with 25 ml of 96% ethanol, thoroughly mixed, and stored in a cooling container during transport to the laboratory, where samples were kept at -25 °C. Macrozoobenthos were collected using the kicking method by Frost et al. (1971), with each collection lasting about 15 minutes and encompassing all present microhabitats. Macrozoobenthos samples were subjected to multiple decantations to remove as much inorganic material and larger non-target organic debris (such as leaves, twigs, and branches) as possible. The cleaned material, free of excess water, was placed into sterile 1 L containers and preserved with 96% ethanol. Samples were kept in a cooling container during transport to the laboratory and subsequently stored at −25 °C. All sample types were processed as soon as possible after collection, with the processing time never exceeding 3 months. All sites were monitored periodically using conventional methods by the Water Research Institute (WRI), which implements the Water Framework Directive in Slovakia (details in suppl. material 1).

The Water Research Institute provided data from conventional methods from 2007 to 2022 (details in suppl. material 2). The material included information on species composition, their respective abundances, calculated individual indices, and an evaluation of the overall ecological status.

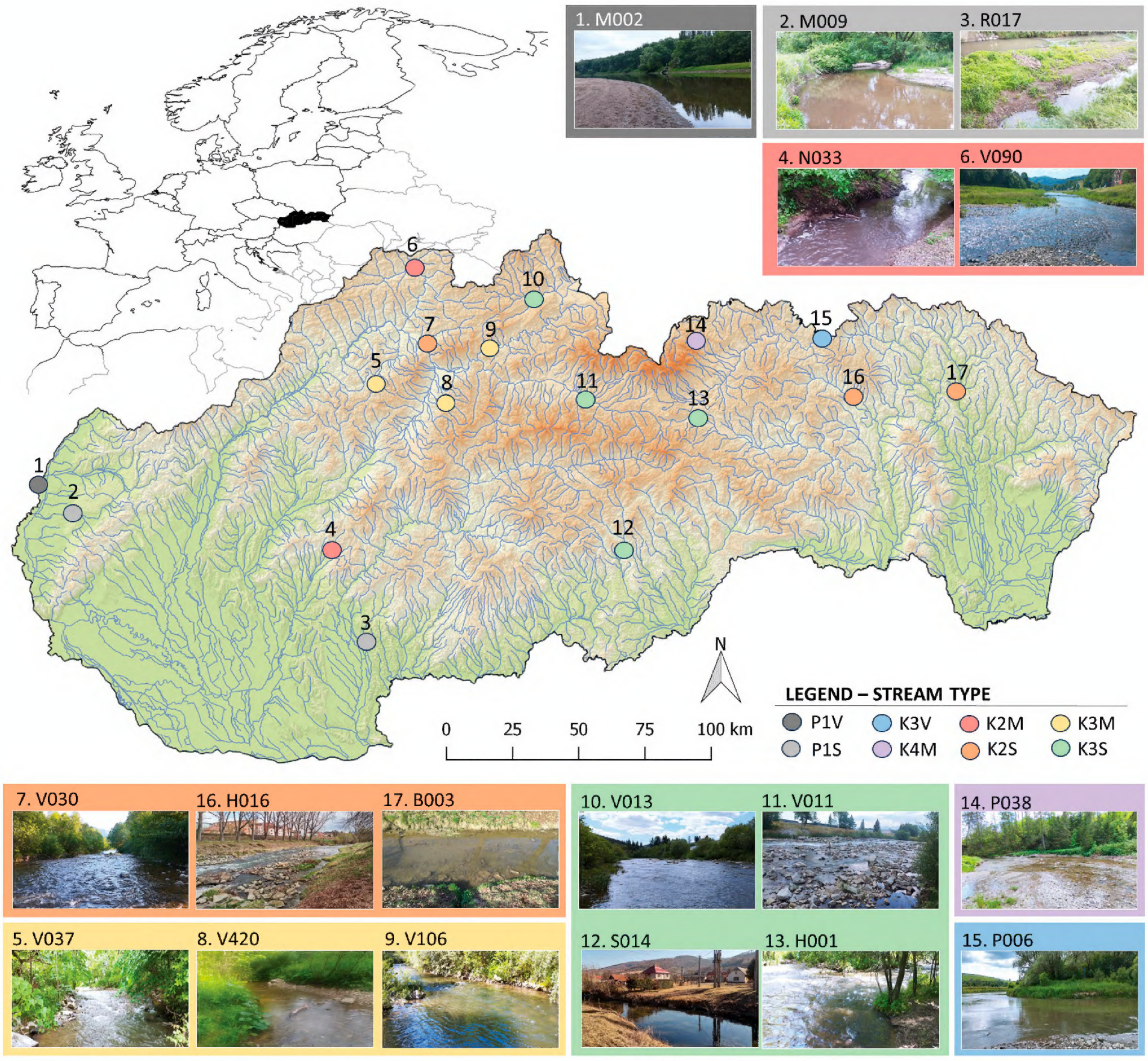

Figure 1. Geographical representation of processed sampling sites across Slovakia, with stream types indicated by coloured circles. The map was generated using QGIS version 2.18.15. Explanation of the stream types: P – Pannonian ecoregion, K – Carpathians ecoregion; 1 - < 200 m a.s.l, 2 - 201–500 m a.s.l, 3 - 501–800 m a.s.l, 4 - > 800 m a.s.l.; M - small streams with area < 100 km^2, S – medium with area 101–1000 km^2, V - large with area > 1000 km^2.

These data were used to compare conventional (CA – conventional approach) and molecular (MA – metabarcoding approach) methods. For detailed information on the sampling and processing of conventional samples, please refer to Hering et al. (2004b) and Makovinská et al. (2015).

DNA extraction, PCR amplification and NGS sequencing

For eDNA water samples, DNA was extracted from the ethanol fixed filters that were torn and dried at 50 °C for approximately 3 hours until all the ethanol had completely evaporated. The extraction and purification were performed using the ReliaPrep™ gDNA Tissue Miniprep System (Promega), following the manufacturer's protocols.

For sediment samples, a combination of phosphate buffer and the DNeasy Blood and Tissue Kit (Qiagen) was used (Taberlet et al. 2012). Briefly, the sediment samples were dried from the 96% ethanol fixative at 55 °C for 3 hours. Phosphate buffer was added to the dried samples at a 1:1 ratio. The samples were mixed using a Multi-Rotator Multi Bio RS-24 stirrer (Biosan) and centrifuged at 4,000 RPM for 30 minutes. The resulting supernatant was filtered through cellulose syringe filters with a pore size of 0.45 µm. The filters were also dried at 50 °C for approximately 1 hour until all the phosphate buffer had completely evaporated, and DNA extraction was performed using the Qiagen manufacturer´s protocol. .

DNA extraction from bulk samples was performed on the whole mixed samples, which included ethanol and partially removed non-target organic and inorganic material (for 90 minutes), to avoid the time-consuming individual selection process. The whole sample was homogenised using a kitchen blender (Electrolux ESB2500, 3 min). Three replicates of 1 ml of homogenised material were taken from each sample and dried at 50 °C for approximately 6 hours until all the ethanol had evaporated. DNA was then extracted and purified using the ReliaPrep gDNA Tissue Miniprep System (Promega) according to the manufacturer's protocol. The genomic DNA from all samples was stored at -25 °C.

Amplification of the target fragment (~420 bp) within the standard barcoding COI marker was carried out using a two-step PCR (for details, see Elbrecht and Steinke 2019). For each isolate, two PCR replicates were always performed, resulting in a total of four PCR replicates for water and sediment samples, and six PCR replicates for bulk samples. The first amplification used BF3 and BR2 primers (Elbrecht et al. 2019), followed by a second amplification with the same primers containing adapters and indexes necessary for sequencing on the Illumina MiSeq platform. At the same time, each 96-well plate contained 10 negative controls randomly distributed. For the detailed composition of reaction mixtures and PCR programs, see suppl. material 3.

The success of the amplification was tested using horizontal electrophoresis on a 1% agarose gel. The concentration of the PCR product was evaluated using the Carestream MI Application program and compared with a DNA standard. The detected concentrations were used to prepare pooled libraries containing approximately equal amounts of DNA from each sample. These libraries were tested again on a horizontal 1% gel electrophoresis, and the desired products were excised and purified from the gel using the Wizard SV Gel kit and the PCR Clean-Up System (Promega). Based on the concentrations of the purified partial libraries, a final library of 20 pM, including 5% PhiX, was prepared and analysed on the Illumina MiSeq with Reagent Kit v3 (2 × 300 bp) at the Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia.

Data processing

Bioinformatic sequence processing followed the pipeline described in Vargovčík et al. (2024). Briefly, the raw sequencing reads underwent demultiplexing, primer trimming (Cutadapt v4.1, Martin 2011), merging (PEAR v0.9.11, Zhang et al. 2013), dereplication, denoising, length filtering, chimera removal and greedy clustering of OTUs at a 97% identity threshold (Vsearch v2.22, Rognes et al. 2016). Besides that, translation filtering after the denoising step (metaMATE, Andújar et al. 2021) and default post-clustering curation (LULU v0.1.0, Frøslev

et al. 2017) were performed. Taxonomy was assigned to the resulting OTUs via BOLD (Ratnasingham and Hebert 2007) using BOLDigger v2.1.0 (Buchner and Leese 2020). Only OTUs with > 85% similarity to their best hit were then retained, focusing on the following animal phyla: Annelida, Arthropoda, Bryozoa, Chordata, Cnidaria, Mollusca, Nematomorpha, Porifera, and Rotifera.

Data analyses

Two datasets were included in the statistical analysis: the first comprised the species composition of 57 samples obtained through CA (the conventional dataset, suppl. material 4), while the second contained the species composition of 17 samples obtained via DNA metabarcoding (the DNA dataset, suppl. material 5). Both datasets were converted into TaXon tables for further analysis using TaxonTableTools v1.5.1 (TTT, Macher et al. 2021b). Within the DNA dataset, PCR and extraction replicates were merged in TTT. After merging the replicates, OTUs with fewer than 10 reads per individual sample type were removed. We also compared the effectiveness of various metabarcoding approaches (bulk samples, eDNA from water, and sediments) using TTT while Venn diagrams, parallel category analyses, and read proportions were generated. Venn diagrams were created using TTT (each conventional collection compared with a bulk sample) to compare the species spectrum of the conventional and DNA datasets. The DNA dataset, including only data from bulk samples, was adjusted according to the requirements of ASTERICS v4.04 (Furse et al. 2006), where various metrics were calculated. Abundance data were replaced by read counts obtained through Illumina sequencing. The read counts were summed for species represented by multiple operational taxonomic units (OTUs) or cryptic taxa, with each species represented by a single Linnaean taxon. The metrics used to calculate Ecological Quality Ratios (EQRs) and the overall ecological status were selected based on stream typology, as defined by Šporka et al. (2009). Specifically, eight stream types and twelve different metrics were used (Table 1). However, these metrics are scored as continuous (e.g., Number of Families, BMWP score) or percentage scores (e.g., Oligo [%] scored taxa = 100%; Gatherers / Collectors [%] scored taxa = 100%), and therefore their values were first normalised, scaling them to a range from 0 (representing bad status) to 1 (representing the reference condition). This normalisation facilitated the integration of metric outcomes (partial EQR values) and the computation of a comprehensive multimetric index (overall EQR value; Šporka et al. 2009) with the following ecological classes: high (1) ≥ 0.8, good (2) ≥ 0.6 to < 0.8, moderate (3) ≥ 0.4 to < 0.6, poor (4) ≥ 0.2 to < 0.4, and bad (5) < 0.2. These data (partial and overall EQR values) were then compared to results obtained using CA (reference values for each stream type are provided in suppl. material 6).

For each site, differences in metric values were expressed within the CA and CA vs. MA comparisons. For each metric, these differences were compared using the Marginal regression model (generalised least-squares - GLS estimation procedure) with two explanatory variables (type of approach, interaction between type of approach and stream type) and the setting of an exchangeable correlation structure within the site. Due to heteroscedasticity, all models were improved by specifying uniform variance within each stream type and different variances between stream types. The marginal regression models were made in R 4.2.1 (R Core Team 2022), using the “nlme” package (Pinheiro et al. 2024).

Table 1. The list of metrics used to calculate the Multimetric Index for eight different stream types (for an explanation of the stream types, see Fig. 1).

Metric	Stream type							
	K2M	K2S	K3M	K3S	K3V (P2)	K4M	P1S	P1V (M1)
Saprobic indices								
Saprobic Index (Zelinka and Marvan)	✓	✓	✓	✓	✓	✓	✓	✓
Oligo [%] (scored taxa = 100%)	✓	✓	✓	✓	✓	✓	✓	✓
Biotic indices								
BMWP Score	✓	✓	✓	✓	✓	✓	✓	✓
Zonation metrics								
Metarhithral [%] (scored taxa = 100%)	✗	✓	✗	✓	✓	✗	✓	✗
Rhithron Typie Index	✓	✓	✓	✓	✓	✓	✓	✓
Index of Biocoenotic Region	✓	✓	✓	✓	✓	✓	✓	✓
Preference of flow velocity								
Rheoindex (Banning, with abundance classes)	✓	✗	✓	✗	✗	✓	✗	✗
Preference of microhabitat								
Type Aka + Lit + Psa [%] (scored taxa = 100%)	✓	✓	✓	✓	✓	✓	✓	✓
Diversity metrics								
Diversity (Margalef index)	✗	✓	✗	✓	✗	✗	✓	✗
EPT taxa	✓	✓	✓	✓	✓	✓	✓	✓
Number of famillies	✗	✓	✗	✓	✗	✗	✓	✗
Feeding functional groups								
Gatherers/ Collectors [%] (scored taxa = 100%)	✗	✓	✗	✓	✗	✗	✓	✗
Number of localities	**2**	**3**	**3**	**4**	**1**	**1**	**2**	**1**

The concordance in classes of ecological status revealed by MA and CA was visualised using a heatmap plot created in "plot.matrix" package (Klinke 2022); the Spearman rank correlation was also calculated, both in R 4.2.1. When repeated measurements of ecological status were conducted at a site, the most frequently observed ecological status class (ESC) was used to assess the coincidence between the two approaches.

Results

Comparative species detection using different DNA metabarcoding samples

Sequencing of the eDNA from water, sediments and bulk samples resulted in 11,713,827 demultiplexing reads from 17 sampling sites. After quality filtering, the reads were clustered into 10,447 OTUs. Nontarget and low-similarity sequences (< 85%) accounted for 9,615 OTUs. By removing negative controls and nontarget taxa (e.g., algae, diatoms, chordates, and terrestrial invertebrates), the final DNA dataset consisted of 625 OTUs of the freshwater invertebrates. Species names were assigned to 589 OTUs, representing 463 species mainly in classes Insecta (343 spp.) and Clitellata (58 spp.). The most species-rich orders within Insecta were Diptera (151 spp.), Trichoptera (60 spp.), Ephemeroptera (56 spp.), Plecoptera (35 spp.) and Coleoptera (28 spp.). Cryptic diversity, indicated by assigning more than one OTU to a single Linnaean species,

was identified in 87 species, with the highest number recorded in the family Chironomidae (23 spp. = 51 OTUs). The species with the most OTUs assigned to them were *Limnodrilus hoffmeisteri* Claparède, 1862 (11 OTUs), *Tubifex tubifex* (Müller, 1774) (9 OTUs), and *Gammarus fossarum* Koch, 1836 (5 OTUs).

Across all sample types (bulk samples, eDNA from water and sediments), we identified an average of 86 species per sampling site, with the fewest number of species captured at site the N033 site (61 spp.) and the highest at site the R017 site (111 spp.). Bulk samples emerged as the most effective method for species detection, as they captured 66 to 96% (∅ 85.03%) of all species present at the sites (45–99 spp.; Fig. 2). In contrast, eDNA from water captured on average 61 fewer species (∅ 25 spp. or 29.87%) per site (ranging from 6 to 53 species, or 6–55%), while eDNA from sediments averaged 75 fewer species (∅ 11 spp. or 13.68%) per site (ranging from 3 to 23 species, or 3–32%) than the bulk sampling strategy. The highest number of species recorded from sediments was at site the V011 site (11 spp.). Notably, the most significant overlap was detected between bulk samples and eDNA from water, averaging 17 overlapping species (ranging from 1 to 33 spp.; suppl. material 7).

The detected species spectrum comprised 15 invertebrate classes, with only 6 (Bivalvia, Clitellata, Copepoda, Hydrozoa, Insecta, and Malacostraca) recorded across all sample types (suppl. material 8). Bulk samples and eDNA from water captured 11 classes with varying class representations, while eDNA from sediments captured 10. Arachnida, Gastropoda, and Gordioida were identified solely from bulk samples, Phylactolaemata was found exclusively in water samples, and Ostracoda appeared in both bulk samples and sediments. Additionally, eDNA from water and sediments captured exclusively three classes (Bdelloidea, Demospongiae, Monogononta). Insecta dominated nearly all samples when examining class representation relative to the number of reads (suppl. material 9). Clitellata were the second most numerous class, particularly prevalent in sediment samples.

Conventional vs. DNA metabarcoding approach in biodiversity assessment

From 2007 to 2022, 57 samples were processed using CA at the monitored localities. The most frequent sampled sites were M002 (13 times), P006 (9 times), V011 (6 times), and H001 (4 times). During this period, 319 species within eight target classes were identified using morphological methods: Insecta (238 spp.), Clitellata (33 spp.), Gastropoda (20 spp.), Bivalvia (12 spp.), Malacostraca (9 spp.), Turbellaria (6 spp.), and Gordioida (1 sp.). The average number of species per sample was 17, with a range of 7 to 31. The most species-rich localities were the submontane streams V420 (2022; 31 species), P006 (2014; 27 species), H001 (2019; 27 species), and V090 (2018; 25 species).

Comparing results from CA with MA (using only bulk samples) showed that, on average, 67 species (∅ 71%, ranging from 35 to 84 spp.) were identified exclusively through bulk samples at all monitored sites. An average of 21 species (ranging from 12 to 30) were identified using the CA at the most frequently monitored locality the Morava River (M002), during the 13 sampling dates. In contrast, 82 species were recorded during a single sampling at that site using the MA. Conversely, at the V420 site, 45 species were identified by the MA, while 37 and 41 species were recorded in two samplings using the CA. The species overlap

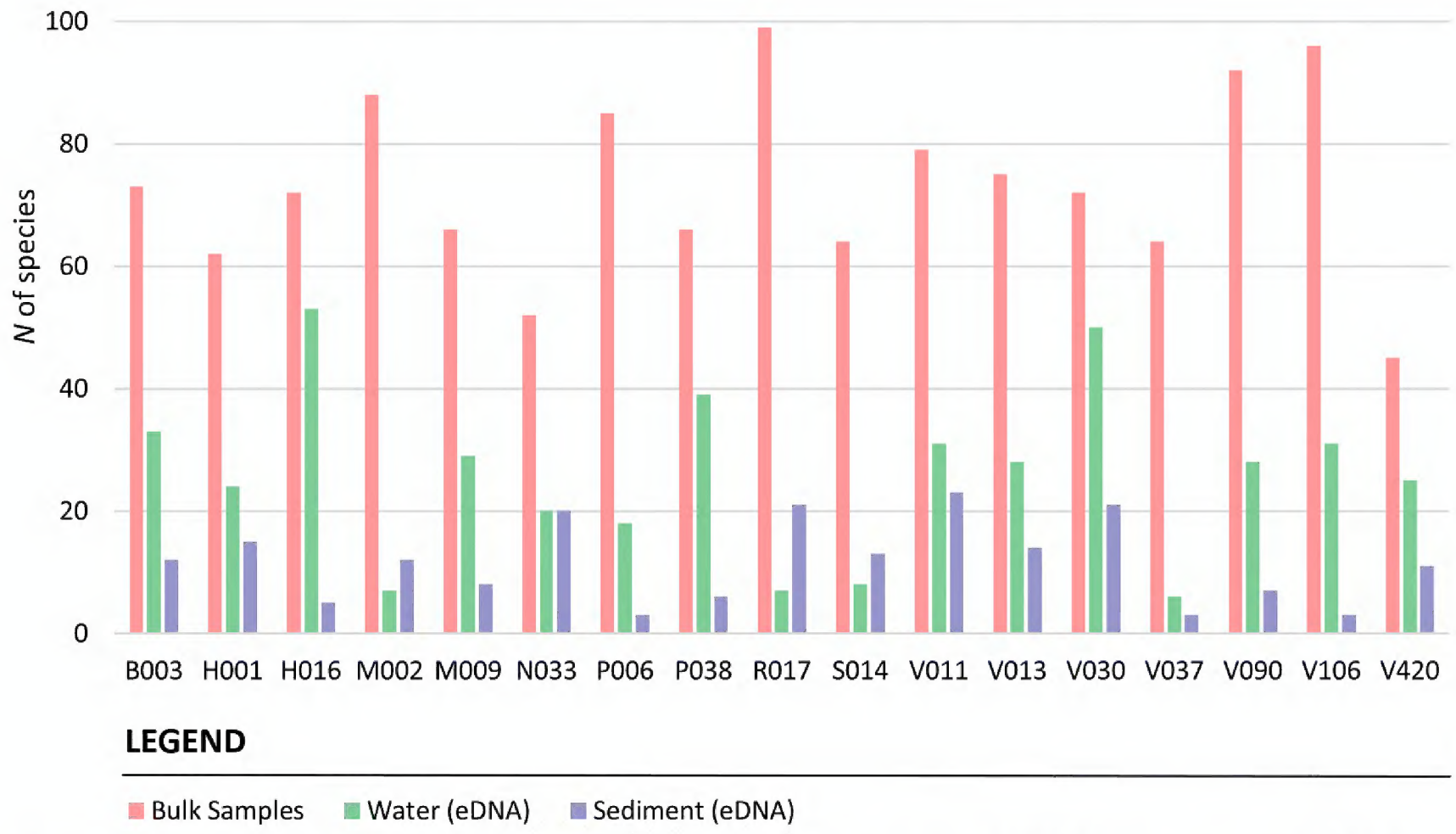

Figure 2. Comparison of three types of metabarcoding samples (bulk samples, eDNA from water, and eDNA from sediments) based on the number of identified macroinvertebrate species.

between both methods (shared species) was only nine species on average per sampling (∅ 10.37%, ranging from 2 to 19; suppl. materials 10, 11).

After combining the 57 conventional samples and 17 samples for DNA metabarcoding, the dataset consisted of 662 records belonging to eight classes, 28 orders, 99 families, 263 genera and 524 species. The overlap between CA and MA was 68% at the family level (65 families) and 52% at the genus level (132 genera; Fig. 3). At the species level, the overlap decreased to 33% (168 species), while 40% (205 species) were identified exclusively through the MA. The orders Myida, Haplotaxida, and Gordioida, each represented by only one species, along with Cycloneritida (two species), Unioida, and Tricladida (three species), were recorded solely by the CA. DNA metabarcoding also identified a single species each from Arhynchobdellida, Decapoda, and Mysida. The more diverse orders were Diptera, Trichoptera, Ephemeroptera, Plecoptera, Tubificida, and Coleoptera. Of the total species captured across both approaches, 80% were identified by the MA and 58% by the CA.

Ecological quality assessment via metabarcoding and conventional approaches

The individual metrics derived from the MA, in most cases, aligned with the ecological quality class (EQC) determined by CA or indicated an improvement in EQC across most stream types (specific values for individual metrics are provided in suppl. materials 12, 13). More pronounced differences between the two methods (change of more than two EQC) were observed mainly among a few samples from larger rivers, particularly Poprad (P1V) and Morava (K3V). However, notable differences were also observed at these sites with higher frequency when measurements were obtained using only CA. Specifically, both methods demonstrated strong agreement in assessing EQC for metrics dependent on

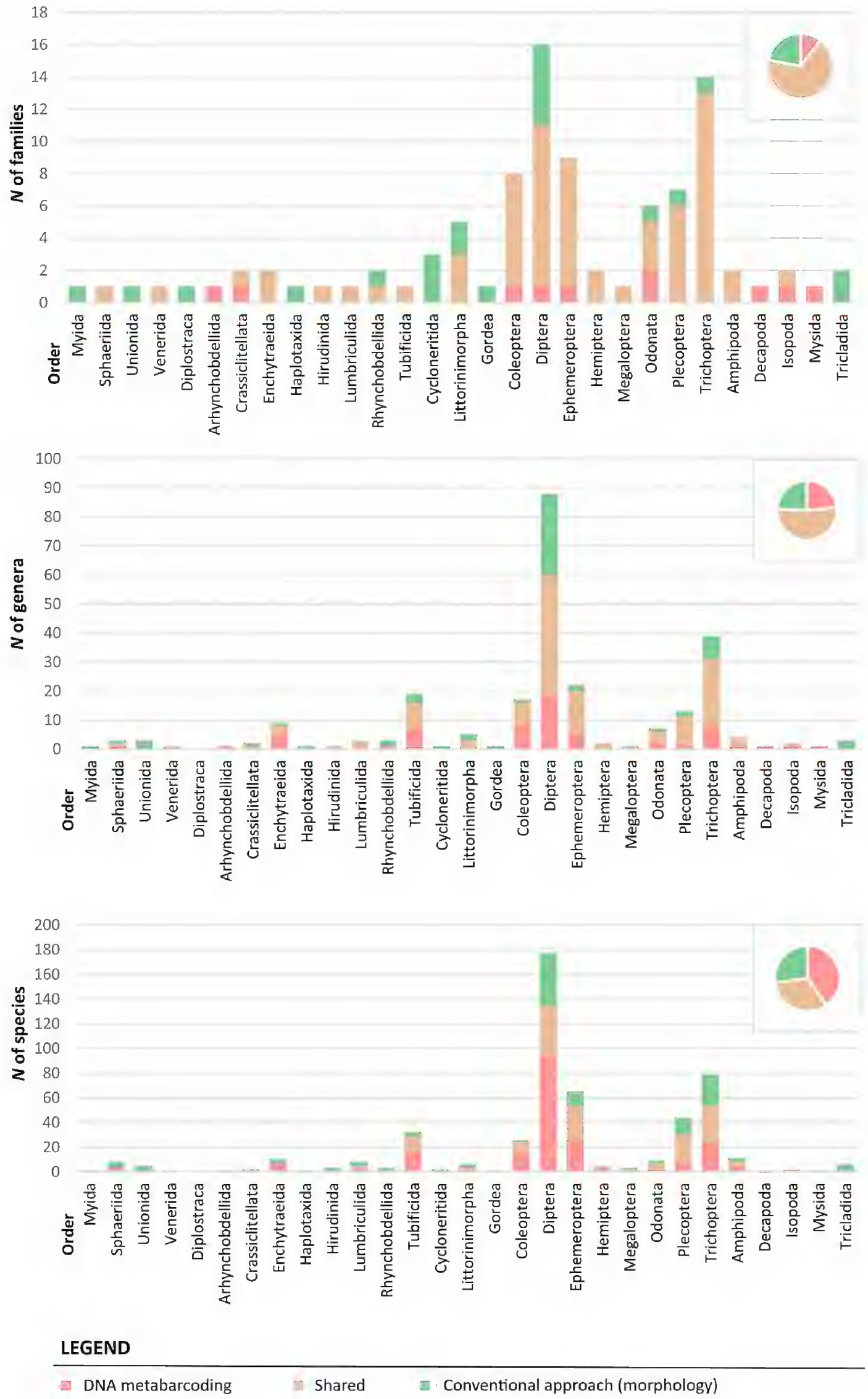

Figure 3. Comparison and detection of overlap between DNA metabarcoding (bulk samples) and conventional approach (morphology) across taxonomic levels (family, genera, and species).

abundances, such as the Saprobic index, Oligosaprobic index, and Rheoindex. Conversely, notable discrepancies were observed in metrics such as EPT taxa, BMWP, and Number of families, which are not directly dependent on abundance (presence/absence data). Usually, these metrics indicated a better EQC based on the MA. The above-described patterns across metrics, including both agreements and discrepancies, were observed across all stream types.

For most metrics, the differences in values obtained within the CA and between the CA and MA were relatively low, typically falling within the range of one EQC (Fig. 4). For the metric "Number of Families," there was a significant difference ($p < 0.05$) both within the CA and between the CA and MA (Table 2). Across sampling dates for each site within the CA, there was most often one EQC class divergence. Comparing the CA and MA EQC assignments, the divergence was at the boundary of one and two EQCs. Regarding the "Margalef Diversity Index," significant differences ($p < 0.05$) between values within the CA and between the CA and MA were observed only for specific stream types. In the K2S stream type, differences mostly stayed within one EQC for both approaches. In contrast, the P1S stream type showed differences mostly within three EQCs for the CA and within two EQCs when comparing the CA to the MA. For the "BMWP index" and "EPT index," significant differences ($p < 0.05$) were found at the method level and also in the interaction between method and stream type. Within the CA, most differences for these metrics were within one or two EQCs, while differences between the CA and MA were generally larger. Specifically, differences in the BMWP index were mainly at the level of two EQCs, whereas for the EPT index, they reached up to three EQC levels.

The final ecological status assessment also reflected the results, as indicated by the EQR values (Fig. 5A, B). At 10 localities, a higher EQC was assigned using the MA compared to the CA. Specifically, four localities shifted from class three (moderate) with the CA to class two (good) with the MA, and six localities shifted from class two (good) to class one (high). At seven localities, the EQC remained unchanged, with two in class one (high), four in class two (good), and one in class three (moderate). In some cases, such as at localities V420, V011, V013, and P006, the EQR values derived from the MA were almost identical to those obtained using the CA.

Discussion

Towards comprehensive macrozoobenthos assessment - bulk samples or eDNA?

Routine monitoring of freshwater ecosystems under the WFD requires international intercalibration to ensure regionally comparable assessments (Birk et al. 2012; Poikane et al. 2014). Reliance on morphological identification of BQEs is time-consuming and prone to variability in taxonomic expertise. Here, we assess the potential of complementing this approach with DNA metabarcoding of macrozoobenthos for a more efficient and consistent ecological status assessment. Regarding the three types of metabarcoding samples, our results align with previous studies (Macher et al. 2018; Hajibabaei et al. 2019; Gleason et al. 2020), indicating that bulk samples captured a greater macrozoobenthos taxonomic diversity, including key EPT bioindicators. Specifically, within three types of metabarcoding samples, we recorded an average of up to ~85% of all macrozoobenthos species at the monitored sites with bulk samples, whereas eDNA from water captured ~30% and sediment only ~14%. Although eDNA samples recovered fewer species, they provide additional insights into taxa that are often underrepresented in bulk samples (Macher et al. 2018; Gleason et al. 2020). Our sediment and water samples exclusively recorded classes such as

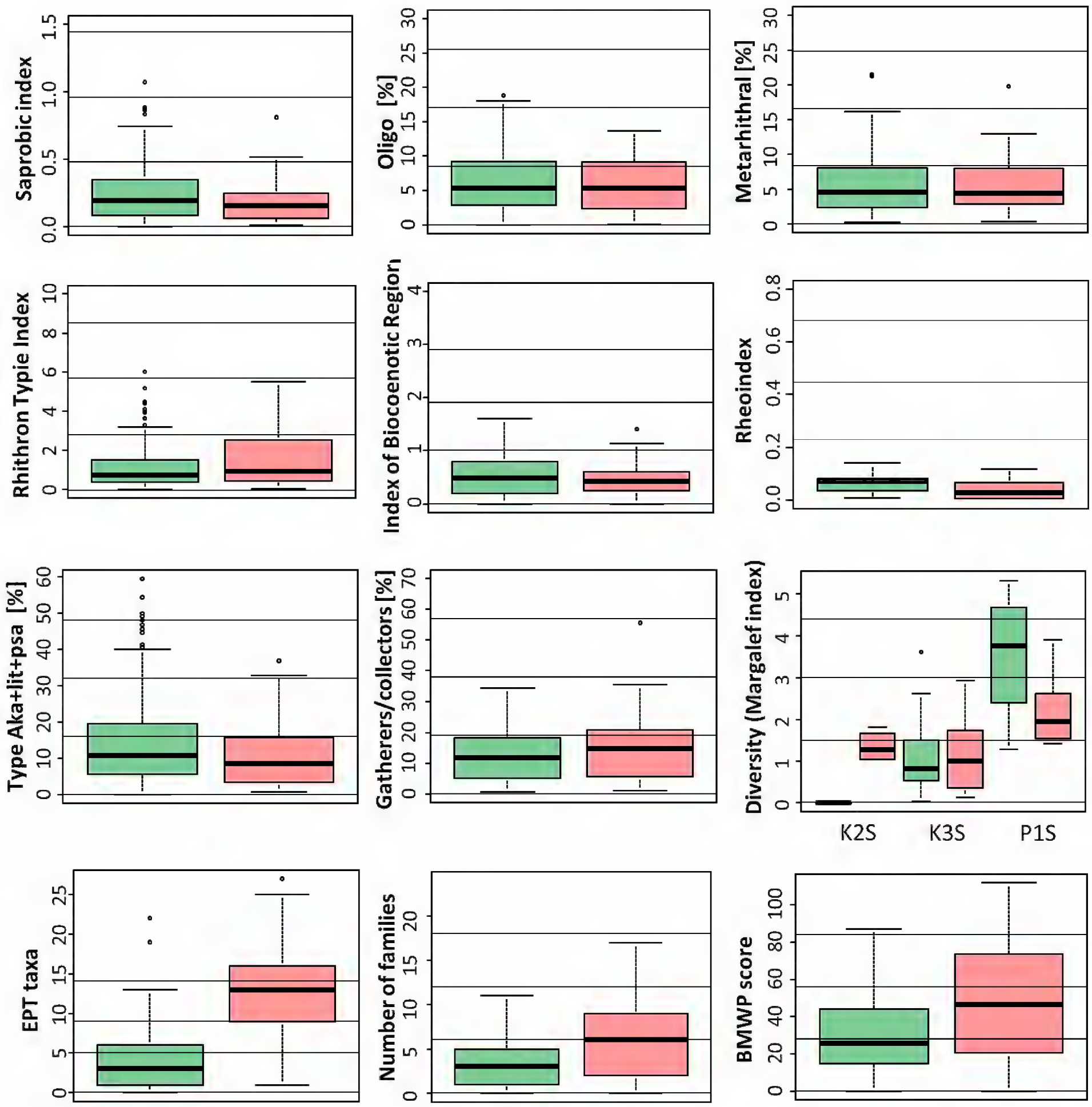

Figure 4. Boxplots showing the variability of within-site differences in metric values obtained using the conventional method and within-site differences in metric values derived from the comparison between the conventional and metabarcoding methods. Vertical lines represent differences in metric values within one up to 4 ecological quality classes. The full name of the metrics is given in Table 1.

Bdelloidea, Demospongiae, and Monogononta, with water samples additionally capturing Phylactolaemata. However, the most significant increase, represented by the number of reads, was primarily associated with representatives of the class Clitellata, with some localities reaching almost 100% of all reads. Similar findings were reported by Xie et al. (2017), highlighting the dominance of Annelids, particularly in the sediment samples. Furthermore, Pawlowski et al.

Table 2. Results of the Marginal Regression Models (generalised least-squares - GLS estimation procedure) comparing within-site variability in differences of metric values obtained using the conventional method and within-site differences in metric values derived from the comparison between the conventional and metabarcoding methods.

Metric	Method				Method: Stream type			Significant difference (p < 0.05)
	demDF	*numDF*	*F value*	*p value*	*numDF*	*F value*	*p value*	
Saprobic indices								
Saprobic Index (Zelinka and Marvan)	193	1	1.202	0.274	12	0.875	0.573	
Oligo [%] (scored taxa = 100%)	193	1	0.554	0.458	12	0.811	0.639	
Biotic indices								
BMWP Score	193	1	32.874	**< 0.001**	12	1.334	0.202	
Zonation metrics								
Metarhithral [%] (scored taxa = 100%)	91	1	1.883	0.173	6	0.904	0.495	
Rhithron Typie Index	193	1	0.852	0.357	12	1.858	0.091	
Index of Biocoenotic Region	193	1	2.705	0.102	12	2.182	0.14	
Preference of flow velocity								
Rheoindex (Banning, with abundance classes)	13	1	1.298	0.275	2	1.693	0.222	
Preference of microhabitat								
Type Aka + Lit + Psa [%] (scored taxa = 100%)	193	1	0.509	0.476	12	0.963	0.486	
Diversity metrics								
Diversity (Margalef index)	48	1	2.453	0.124	4	4.262	**< 0.005**	clas_K2S: metabar_K2S, clas_P1S:metabar_P1S
Feeding functional groups								
Gatherers/ Collectors [%] (scored taxa = 100%)	48	1	0.866	0.357	4	0.269	0.896	
Species richness								
Number of famillies	48	1	4.072	**0.049**	4	0.713	0.587	
EPT taxa	193	1	471.868	**< 0.001**	12	4.47	**< 0.001**	clas_K2M: metabar_K2M, clas_K3M: metabar_K3M, clas_K2S: metabar_K2S,clas_K3S: metabar_K3S, clas_P1S:metabar_P1S, clas_P2:metabar_P2, clas_M1:metabar_M1

(2022) observed that soft sediments predominantly reflect meiofauna and microbial taxa with a limited representation of macrozoobenthos.

Spatially, bulk samples provide a more localised and detailed representation of site-specific biodiversity, whereas aquatic eDNA offers a broader view of watershed-level dynamics, including stress regimes and temporal shifts in species composition (Macher et al. 2018). This duality highlights the feasibility of combining both methods: bulk samples as a cornerstone of biomonitoring and eDNA as a complementary tool to capture underrepresented taxa and assess ecological trends at large scales. For example, our eDNA water samples, like Múrria et al. (2024), uniquely detected organisms restricted to the water column, such as Bdelloidea and Monogononta, which were often absent in bulk samples. Furthermore, the advantage of integrating the two methods was supported by the identification of rare and cryptic taxa, particularly within

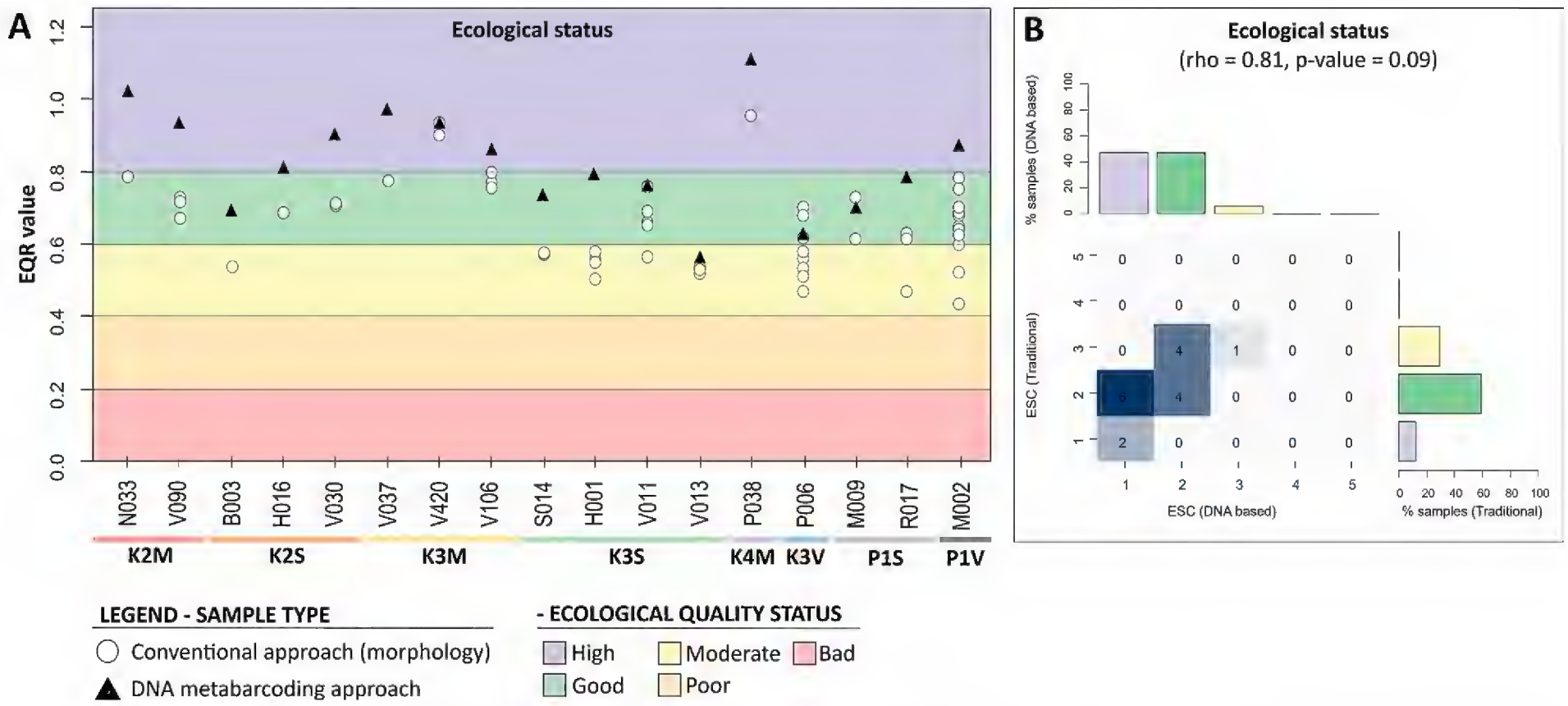

Figure 5. A. The final ecological status assessment, represented by EQR values derived from the conventional approach (57 samples) and the DNA metabarcoding approach (17 samples) across 17 localities and eight stream types. **B.** Metrics comparing ecological status classes of the macrozoobenthos dataset obtained using DNA-based (DNA metabarcoding) and traditional (conventional) approaches. The proportions of samples per status class are displayed as bar charts. Spearman correlation was used to evaluate the relationship between the two methods.

taxonomically challenging groups such as Chironomidae and Clitellata. Cryptic diversity in the case of this study was most pronounced within the family Chironomidae, where 23 Linnaean species were represented by 51 OTUs.

An integrated approach would allow for a more comprehensive and accurate assessment of freshwater ecosystems, which is in line with the broader objectives of the WFD. On the other hand, it is more time-consuming compared to an eDNA only analysis. Given the increasing environmental pressures and the need for more reliable and standardised biomonitoring tools, the pros and cons of each approach or data produced will need to be weighed and a methodology chosen that will meet the requirements of reliable monitoring and its effective implementation.

DNA metabarcoding vs. conventional approach: strengths, gaps, and overlap

Besides labour and time efficiency, one of the most compelling arguments for integrating DNA metabarcoding into routine biomonitoring is its higher efficiency in detecting a broader species spectrum compared to CA, as demonstrated by numerous studies across various aquatic ecosystems (Andújar et al. 2017; Beermann et al. 2018; Gleason et al. 2020; Duarte et al. 2023; Van den Bulcke et al. 2024). Focusing on macrozoobenthos as probably the most informative bioindicator group, Macher et al. (2025) reported that up to 61.5% of all present species were recorded exclusively using the MA, 11.9% by the CA and 26.5% using both methods (shared species). Here we detected slightly lower rates, with 40% of species recorded exclusively by the MA, 27% by the CA, and 32.8% shared between both methods. These differences may partly reflect changes in environmental conditions (e.g., temperature, flow, microhabitat composition) at the studied sites, as well as temporal changes that caused shifts in species composition, since our comparison included 17 DNA metabarcoding samples

(one per site) and 57 conventional samples collected over 15 years (2007–2022). However, it is important to acknowledge the potential limitations of DNA metabarcoding, such as the occurrence of false positives that can artificially inflate the number of OTUs and thus the overall taxon list. These may arise from the presence of nuclear copies of mitochondrial genes (NUMTs; Schultz and Hebert 2022), errors or inconsistencies in reference databases (including synonymous species names or misidentified sequences; Baena-Bejarano et al. 2023; Šamulková et al. 2025), and technical issues during sequencing or amplification (for synopsis, see Fueyo 2024c). Many of these problems currently lack definitive solutions, but in our study, we attempted to mitigate some of these issues by building local reference databases (AquaBOL.SK), carefully verifying the obtained species spectrum against freshwater invertebrate checklists for Slovakia (Šporka et al. 2003) and filtering out all OTUs with fewer than 10 reads.

Building on this, when comparing the species spectrum captured in a single DNA metabarcoding sample to a single morphology-based sample using the CA, the results consistently show that DNA metabarcoding detects more species, regardless of stream type or year of sampling. For example, at site M002 (Morava River), 13 samples were collected using the CA, with 12 to 30 species identified per sample, while a single DNA metabarcoding sample recorded up to 82 species. However, the most relevant example is site S014 (Rimava River), which was sampled simultaneously, and the difference between both approaches was up to 37 species in favour of DNA metabarcoding. It is also important to note that the significant increase in the number of recorded species by the MA was likely due to the non-attendance of the "subsampling" approach and the analysis of the entire collected material.

Despite its broader species coverage, DNA metabarcoding was less effective in identifying certain groups, such as Gastropoda and Bivalvia, consistently with other studies (e.g., Van den Bulcke et al. 2024). On the other hand, DNA metabarcoding can more clearly distinguish representatives of the order Diptera (Beerman et al. 2018; Macher et al. 2025), which is particularly challenging for precise morphological identification. In this study, the number of Diptera species captured by DNA metabarcoding was more than double that of morpho-taxonomy, and this trend was also confirmed in other orders, such as Ephemeroptera, Coleoptera, Tubificida, and Enchytraeida.

Perhaps the most surprising finding across several studies is the relatively low taxonomic overlap between the two approaches (CA vs MA). For example, Macher et al. (2025) reported only 26.5% overlap (shared taxa), Vasselon et al. (2017) found 13% overlap for diatom species, Van den Bulcke et al. (2024) reported 34%, and Duarte et al. (2023) found 23% overlap for marine benthic invertebrates. We also examined the overlap between these methods at three specific taxonomic levels (family, genus, and species), revealing a clear decrease with decreasing taxonomic rank (69% overlap at the family level, 52% at the genus level, and only 33% at the species level). Conversely, the number of taxa recorded exclusively through DNA metabarcoding increased with decreasing taxonomic rank (10% at the family level, 24% at the genus level, and 40% at the species level), reflecting limitations of morphological identification (Haase et al. 2006; Gleason et al. 2020). However, it is important to note that the incompleteness of reference databases can also significantly influence these results (Vasselon et al. 2017), potentially leading to changes in the number of shared

taxa, either decreasing or increasing. Additionally, variation introduced by the sampling process itself can affect these findings. Due to limited replicates in our study, we could not directly compare sampling variability between CA and MA, but previous studies have documented considerable variability in traditional sampling (Ramos-Merchante and Prenda, 2017; Seidel et al. 2022), which should be taken into account when interpreting overlap percentages.

The perspective of DNA metabarcoding data in Multimetric Index calculation and ecological quality assessment

Improving biodiversity assessment and monitoring is probably the key reason for efforts to apply DNA methods in these processes. However, in evaluating water bodies, one of the significant challenges of metabarcoding is to provide reliable abundance data, which is essential for abundance-dependent metrics (Elbrecht and Leese 2015; Pinol et al. 2015).

Essentially, the only solution is to use the number of reads generated by NGS sequencing as a proxy for abundance. Although this approach is often considered controversial due to biases introduced by PCR amplification or primer specificity (Doi et al. 2017; Krehenwinkel et al. 2017; Shelton et al. 2023); however, it could still provide usable information. The number of reads has its limitations, e.g., it does not distinguish the life stages of organisms, which may be crucial in specific cases. Our results suggest that despite the stochasticity of the processes, read numbers may be usable. Moreover, such positive correlation between the number of reads and the species abundance has already been demonstrated in several studies (e.g., Deagle et al. 2019; Di Muri et al. 2020; Salis et al. 2024). Another possible solution is to transform presence/absence data to abundance (1/0) data as in Buchner et al. (2019), who showed that EQC class remained unchanged in 76.6% of cases, decreased by one class in 12%, and improved by one class in 11.2%. However, since this approach can significantly distort the actual situation, we decided to give a chance to the number of reads and analyse their ability to reflect the abundance of the species present. We hypothesised that abundance-dependent metrics would show significantly different values than those obtained using conventional methodologies, but the comparison at the method level (conventional vs. conventional, conventional vs. metabarcoding) was encouraging in that (i) no or low change in EQC occurred for abundance-dependent metrics (e.g., Saprobic Index, Index of Biocoenotic Region), (ii) significant change was observed in some cases by up to 3 EQC for abundance-independent metrics (e.g., EPT taxa, Number of Families and BMWP Score) and (iii) significant differences between individual stream types were demonstrated only for Diversity (Margalef Index) and Rheoindex. Our testing supported the assumption that read counts can potentially replace classical abundance data. Still, their use needs to be tested in more detail, and the use of some metrics in ecological status assessment will need to be reconsidered. It seems that metrics based on presence/absence data (e.g., BMWP Score, EPT Taxa, Number of Families) are more problematic because they have demonstrated significant differences between CA and MA. Similar findings were also reported by Múrria et al. (2024), where the application of metabarcoding data from bulk samples to the IBMWP index (Iberian Biological Monitoring Working Party index) led to an improvement in EQC at

two of the five studied sites. At the remaining three sites, the EQC remained unchanged, and the index value derived from the MA was lower than that from the CA at only one site. In another study from Spain performed by Fueyo et al. (2024b), it was demonstrated that molecular methods (bulk and eDNA from water) showed correlations with morphological identification for EQR values of the IBMWP index. However, differences were observed in the estimated ecological status of the rivers, with bulk samples tending to indicate a higher status. Additionally, a recent comprehensive study from Germany (Macher et al. 2025), which included a wider range of indices, confirmed that applying metabarcoding data improved the EQC at nearly one-third of the surveyed sites. However, in most cases, the EQC remained unchanged, further highlighting the high potential of DNA metabarcoding for routine monitoring.

We can expect even more significant differences if reference barcode databases, such as BOLD, continue to improve species coverage. This was supported by Fueyo et al. (2024a), who also reported an improvement in the ecological quality class based on the non-abundance metric (IBMWP) in 16% of samples after incorporating their newly obtained sequences into the BOLD database. Their study highlights the importance of contributing to and developing regional reference databases, which can enhance the detection and identification of novel taxa, thereby improving the accuracy of ecological status assessments derived from metabarcoding data. It is worth mentioning that, especially in Central Europe, barcode reference databases have recently been significantly improved (Morinière et al. 2017; Macko et al. 2024; Vuataz et al. 2024), which may have influenced our results. Conversely, significant gaps in these databases still remain (e.g. Csabai et al. 2023). Finally, our knowledge of the true diversity of insects, even in a “well-studied” region such as Europe, remains limited, as demonstrated e.g. by the metabarcoding study of Buchner et al. (2024). Recent studies repeatedly highlight the need to build regional reference databases, which ultimately play the most crucial role in the broader integration of DNA metabarcoding into biomonitoring. On the other hand, despite the gaps in the databases, our results, along with those of similar metabarcoding studies (Múrria et al. 2024; Macher et al. 2025), demonstrate that the assessment of the ecological status of freshwater ecosystems using the metabarcoding approach is faster and can be more efficient than the conventional methodology used so far.

Regarding observed improvements in ecological status within this study, this could be a matter of the reference values. They are essential for calculating individual metrics but originally were determined based on CA. Implementing DNA metabarcoding into routine biomonitoring will likely require their re-evaluation, which could significantly reduce discrepancies in the resulting assessments between the two approaches.

Conclusion

Assessing the ecological status of aquatic ecosystems is essential for their conservation, restoration, and sustainable management. Current practices underline the urgent need for innovative approaches to make this time-intensive and costly process more efficient. Our study confirms the potential and feasibility of using DNA metabarcoding for routine monitoring within the context

of WFD implementation. Among the three sample types tested (eDNA from water, sediment, and bulk samples), the bulk samples detected the most comprehensive species spectrum. However, we recommend incorporating eDNA from water and sediment into monitoring programs, as these sample types reveal unique components of species composition not captured by bulk samples. DNA metabarcoding demonstrates clear advantages in terms of efficiency and comprehensiveness, especially in detecting taxonomically challenging groups such as Diptera and Clitellata.

The analysis of individual metrics shows that using the number of reads as a proxy for abundance can yield results comparable to conventional methods for abundance-dependent metrics. However, metrics reliant on presence/absence data, such as BMWP scores or EPT taxa, revealed significant differences due to DNA metabarcoding's enhanced species detection capabilities. This highlights the need to calibrate such metrics to align with the broader detection range of this method. Furthermore, expanding and refining reference databases remains essential, as this would likely enhance the detection of even more species and improve ecological assessments. Despite current challenges, our findings suggest that DNA metabarcoding is already a viable alternative to conventional approaches in routine biomonitoring. Differences in the estimation of ecological state classes could hopefully be resolved by revising the thresholds that are considered in the interpretation of the metric values but have been set based on and for data from conventional procedures. With increasing environmental pressures on freshwater ecosystems, the adoption of this innovative method has the potential to significantly improve biodiversity assessment rates. This, in turn, could facilitate more effective conservation and management practices, supporting the objectives of the WFD and contributing to the sustainable management of aquatic ecosystems. However, as acknowledged by previous studies, including our own, further research is needed to address the known limitations and challenges of these techniques.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Use of AI

No use of AI was reported.

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Author contributions

Conceptualization: MŠ, PM, FČ. Data curation: MŠ. Resources: MŠ, PM, EME, ML. Formal analysis: PB. Methodology: MŠ, PM, OV, KT. Project administration: ZČZ. Supervision: FČ. Validation: FČ. Visualisation: MŠ, PB, PM. Writing – original draft: MŠ, PB, PM, FČ. Writing – review and editing: OV, KT, ZČZ, ML, EME.

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Data availability

Additional data are in supplementary materials on FigShare (https://figshare.com/) under https://doi.org/10.6084/m9.figshare.29269859 or available on request.

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